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Apoptosis of Tumour Cells by Temperature and Anti-tumour Drug: Microscopic and Macroscopic Investigations

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Abstract: Human ovarian carcinoma cell line (COCL) behaves differently under different temperatures and/or anti-tumour drug Carmofur levels, which result in metabolic and ultrastructural changes of the tumour cells. The technique of microcalorimetry is adopted to investigate the metabolic behaviour of the tumour cells; while the technique of transmission electron microscopy (TEM) is used to detect cells' ultrastructural changes. Quantitative analysis is also performed through image processing. The mechanisms of hyperthermia and chemotherapy in treatment of tumour cells are explored at both macroscopic and microscopic levels. It is shown that a high temperature reduces metabolic activity of the tumour cells, appears to cause both apoptosis and necrosis in COCL, and results in a series of malignant changes in the cell morphology. The concurrent application of hyperthermia and chemotherapy can speed up the apoptosis of the tumour cells and reduce cells' metabolic power. A sustained and longer action of such a concurrent application will lead to ultimate death of the tumour cells.

Keywords: hyperthermia, microcalorimetry, TEM, apoptosis, tumour cells

1. Introduction

Hyperthermia is usually applied as an adjunct to an already established treatment modality, as it can increase the effectiveness and decrease the toxicity of currently available cancer treatment such as chemotherapy and radiation. Even a mildly elevated temperature is known to significantly potentiate the effects of radiotherapy and chemotherapy (Wiedemann GJ 1993; Zhu WG 1995; Rumi Murata 2001). Such combinations of treatment modalities could result in lower doses of chemotherapeutic agents or radioactivity necessary to achieve a given effect.

Investigations have demonstrated that hyperthermia could affect the stability of proteins and induce the synthesis of heat shock proteins (Lepock JR 1983; Lepock JR 1993; Senisterra GA 1997; Freeman ML 1999). Hyperthermia could also induce various changes of cell membrane and cytoskeletal organization (Coss RA 1982; Coss RA 1996; Ronald A 1996). Recent publications have focused on the effect of hyperthermia on distinct cellular signalling pathways, in particular, of those involved in "heat shock response", cell cycle regulation, and apoptosis. Furthermore, hyperthermia influences tumour blood flow, oxygen and nutrient supply, as well as the cellular immune response under vivo conditions (Sakaguchi Y 1995; Matsumoto H 1997; Burd R 1998; Bert Hildebrandt 2002)

Several different modalities of hyperthermia are currently used in clinical treatment with satisfactory results (Moroz P 2001). Improved response and survival rates have been observed in patients treated with hyperthermia and radiotherapy compared with radiotherapy alone in several comparative phase-III trials (Vernon CC 1996; Bert Hildebrandt 2002; J van der Zee 2002). But

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all hyperthermia modalities have in common that their efficacy is not enough to replace any one of the established therapy modalities when applied alone. An unequivocal identification of the mechanisms leading to favourable clinical results of hyperthermia has not yet been obtained for various reasons (Engin 1996; Bert Hildebrandt 2002).

However, successful applications of hyperthermia in clinical cases require a detailed understanding of the exposure conditions such as temperature, length of heating time, and the dose of antitumour drugs. Microcalorimetry is a powerful investigative tool to assess the effects of various exposure conditions. It can be adopted to measure the metabolic activity of living cells directly and quickly due to the fact that all metabolic courses inside the living cells are accompanied with energy transmission and heat transfer. Calorimeters with sufficient sensitivity can be used to monitor the heat produced by the living cells. There have been many reports on applications of microcalorimetry in investigation into the tumour hyperthermia mechanism. The results indicate that a high temperature reduces metabolic activity of the tumour cells and induces cell apoptosis, and the combined use of the chemotherapy and hyperthermia has a synergistic antitumour effect (Feng Y 1997; G. Kehrer 1999; Liu Yuwen 2001; Hui Zhou 2002; Liu Yuwen 2002).

Many references can be found on the subject of apoptosis, ranging from the early descriptions with transmission electron microscopy (TEM) (JF Kerr 1972; Warner 1972; Wyllie AH 1980; Wyllie AH 1999; Blankenberg FG 2000) to analysis of highly specific factors in the apoptotic pathway in situ with molecular probes (de jong L 1996; Gall JG 1999; Bert Hildebrandt 2002). The commonly used techniques for apoptosis study are summarised in Willingham (Willingham 1999). TEM is the best method to observe cellular changes in ultrastructure during apoptotic progression, especially for distinguishing it from necrosis, as these processes may share common features (Ying-Bo Shui 2000; Coby Meijer 2001; Marian L. Miller 2002). The metabolism varies in response to temperature variation, addition of anti-tumour drugs, and synergistic effects of temperature and drugs. The combined application of microcalorimetry and TEM will provide us with a powerful tool to obtain useful information about the activity of the total metabolism of the living tumour cells, and to explain the results at the ultrastructural level. However, there has been very limited number of reports in the open literature on the investigation (Li L 2002). This paper will contribute to this subject.

This work aims to investigate the effect of hyperthermia and anti-tumour drug Carmofur on human ovarian carcinoma cell line (COCL). The technique of microcalorimetry is applied in measurement of the metabolic thermogenic curve of the tumour cells; while the technique of TEM is adopted to detect the changes in cell's ultrastructure during apoptotic progression. Quantitative analysis is also performed through image processing. The mechanisms of hyperthermia and chemotherapy in the treatment of the tumour cells are explored at both macroscopic and microscopic levels. The results reveal that hyperthermia induces apoptosis of tumour cells. Hyperthermia changes the arrangement of cell cytoskeleton and makes the cytoskeleton disordered and incomplete, leading to functional damage and ultimate death of the cells.

2. Experimental

2.1 Experimental Materials

The cell line used in this investigation was the human ovary cell line, which was kindly provided by China Centre for Type Culture Collection, Wuhan University, P. R. China.

DMEM was purchased from Sigma Inc.USA. The fetal calf serum was provided by Hangzhou Evergreen Biological Materials Pty Ltd. Penicillin and streptomycin were obtained from North China Pharmaceutical Plant.
Cells were cultured in DMEM medium supplemented with 10% fetal calf serum, 100IUml^{-1} penicillin and 100 gμml^{-1} streptomycin (pH = 7.2-7.3) in a humidified atmosphere of 5% CO_2 at 37.0°C. The same medium was used for the calorimetric experiments.

The anti-pernicious tumour drug made in Japan is called Carmofur (HCFU in short). Its molecular structure is $C_{11}H_{16}FN_3O_4$.

2.2 Experimental Apparatuses

The ultrastructural changes of the tumour cell were observed using a JEM-100CX transmission electron microscopy (TEM). The accelerating voltage used was 80kV. Typical cell images were chosen under TEM and were recorded in films at 2900×.

The microcalorimeter used in this work was LKB 2277 Bioactivity Monitor, which was a new type of heat-flow microcalorimeter and had a very high sensitivity with the detection limit of 0.15μW and the baseline stability (over a period of 24h) of 0.2μW. It was used to obtain the power-time curves. It can monitor continuously a wide variety of processes and complex systems over a temperature range 20-80°C. The LKB 2210 recorder was used in this experiment for continuous recording of the power-time curves for growth. More details of the performance and specifications of the instrument can be found in (Suurkuusk J 1982; Xie CL 1988).

2.3 Experimental Design

Cell cultivation: Cells to be tested were put in a T-25 plastic cell culture flask containing 8 ml medium according to the routines, and then were supplemented with 5% CO_2. The temperature of the cultivate cells was maintained constant at 37°C.

The cells were collected in their exponential growth phase and the cell concentration was adjusted to 3.28×10^5cells·mL^{-1} by adding the medium. Then in the same growth atmosphere, took 1 mL cell suspension and put it into 3mL ampoule. At the beginning of the experiment, 0.5μg·mL^{-1} Carmofur was added into the ampoule with supplement of 5% CO_2. Put the testing ampoul of 1 mL culture fluid and the reference ampoul of 1mL sterilis culture medium into the microcalorimeter to monitor the growth of the cells. The growth metabolic thermal power curve of the COCL was recorded at a paper moving at the speed of 0.1mm/min.

Measurements were carried out at 37°C corresponding to the physiological body temperature, 40°C, 42.5°C, and 43.5°C, respectively. A comparison group is set at each temperature point. At each temperature, cultivate COCL under 5% CO_2 for 23 h, and then collect cells, take cell precipitate fluid, and finally make TEM samples.

It is worth mentioning that the use of 41.5°C and 43.5°C is interesting from an experimental perspective. However, using such temperatures for 23 hours and longer is not clinically realistic.

2.4 Preparation of Tumour Cell Samples for TEM

TEM samples of COCL were prepared according to the routines. The cell precipitate fluid is fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.4) overnight at 4°C, after washing in same buffer twice, postfixed in 1% osmium tetroxide for 1h, then dehydrated through a graded

alcohol series (30% - 100%), 100% alcohol dehydrated twice. The samples were embedded into Epon812 at 60°C for 24 more hours. The ultra-thin sections were stained with uranyl acetate and lead citrate. The sections were observed using the JEM 100CX-II TEM.

2.5 Image Processing

Actually, the images observed under TEM are the cross-section of cells. We chosen typical cell morphology and taken about 20 photos at 2900× for each of the comparison and test groups. The photos were imported directly into an image processing unit (made in German by IBAS) using a camera recorder, and were displayed on the monitor. Every cell and cell nuclei are separated from original image using manual segmentation method, which is considered as the most accurate approach in this kind of measurement (Robb 1995). Because the cell boundary on the TEM photos is very clear, the boundary can be traced reliably by a trained operator. With the traced cell boundary, the area of each cross section and changes in the cross sections can be easily calculated, which can be used to infer the changes in cell and nuclei volumes, and nucleus to cytoplasm (N/C) ratio. Analyse 30 to 80 cells statistically for each group. Plot these quantitative results versus temperature.

3. Results

3.1 Effects of Temperature

The power-time curves for COCL at 37°C, 40°C, 42.5°C, and 43.5°C are respectively depicted in Fig.1. Fig1. shows that the thermal power of the cells decreases with the increase of temperature except at around 40°C. It was found without a satisfactory explanation that the thermal power of the cells seemed usually higher at 40°C than that at 37°C during 15 hours. Meanwhile, at 40°C, there was a relatively small difference between the power-time curves of the test and comparison groups. However, increase of the temperature to above 40°C results in a significant difference in the power-time curve between the test and comparison groups, suggesting that the inhibiting effect of temperature on COCL becomes more significant.

3.2 Cooperative Effects of Both Temperature and HCFU

Through viable count, the relationship of viable count versus time were obtained under 0.5μg.mL^{-1} HCFU for COCL at 37°C, 40°C, 42.5°C, and 43.5°C, respectively. The results are shown in Fig.2.

Fig.2 shows that the highest number of cells is obtained in the comparison group at 37°C and without addition of HCFU, revealing that a higher temperature with or without HCFU can inhibit the growth of tumour cells. This agrees to the results from the analysis of the power-time curves shown in Fig. 3 under the same conditions, as will be detailed later in this subsection. Meanwhile, Fig. 2 also shows that at all test temperatures, the addition of HCFU gives better results and thus enhances the damage of tumour cells. This implies that the use of HCFU is beneficial in killing tumour cells.

It is seen from Fig. 2 that there is no significant change in viable counts in the first 23 hours. Applications of high temperature and/or HCFU for sufficiently long time will result in a significant decrease of the viable counts, implying the death of a large number of cells. When temperature reaches 43.5°C, the COCL cannot grow anymore. The higher the temperature and the longer the time, the more significant the killing effect on the tumour cells.

The power-time curves for COCL growth at 37°C, 40°C, 42.5°C, and 43.5°C with 0.5μg.mL^{-1} HCFU are respectively shown in Fig.3.

It is seen from Fig. 3 that for all test groups with or without addition of HCFU, the COCL metabolic power-time curves are below that of the comparison group at 37°C. An investigation of the metabolic power-time curves for all test groups with addition of HCFU shows that the anti-tumour drug of HCFU has a strong inhibiting effect on the COCL. Previous studies have indicated that the combined use of hyperthermia and HCFU has a synergistic antitumour effect (Liu Yuwen 2001; Liu Yuwen 2002). High heat can strengthen the sensibility of the antitumour drug to the tumour cells. It can increase the concentration of the chemotherapeutic drug within the tumour cells, increase their chemical reaction rates, and strengthen the cellulotoxic effect as well. Heat can also reduce the repair ability of the sublethal damage of the tumour cells on the chemotherapeutic drugs (Henle KJ 1978; Li GC 1980; Engin 1996). Another advantage of the cooperation of hyperthermia and chemotherapy is that it can reduce the dosage of the chemotherapy drug. In clinic applications, high temperature may have some side effects on the normal cells, therefore cooperation of mild-temperature-hyperthermia plus chemotherapy is more viable and more practical.

3.3 Analysis of TEM Results

Figs. 4 through 7 show the ultrastructure morphology of the COCL for the comparison groups at 37°C, 40°C, 42.5°C, and 43.5°C, respectively.

Fig. 4 for 37°C shows that the cells are tightly connected with clear boundaries. The structure of organelles is normal. The cell nuclei are big with a high nucleus/cytoplasm ratio. No cell apoptosis is observed.

In Fig.5 for 40°C, cell volume becomes smaller compared with that at the temperature of 37°C. Connections among cells become loose with spaces present. The outer microvillus of the cells begins to separate. It is also seen that cell nuclei contain one or more nucleoli; and some nucleus membranes have high plication.

It is seen from Fig.6 for 42.5°C that there are big spaces among the cells. Nuclei present polymorphic or deformed shape with high plication in nucleus membranes, chromatin condensation, nucleus fragments of varying size, loss of integrity of plasma membrane and organelles. This implies the apoptosis of tumour cells. But in some cells swollen nucleus and disrupted plasma membrane are observed that like advanced necrosis. These phenomena reveal that a high temperature appears to cause both apoptosis and necrosis in COCL.

Fig.7 for 43.5°C shows that COCL plasma membrane was broken; the structure of the organelles disappeared; and the nucleus was broken into pieces. This implies the death of the cells.

Figs. 8 through 11 depict the ultrastructure morphology of the COCL for the test groups at 37°C, 40°C, 42.5°C, and 43.5°C, all with 0.5μg.mL^{-1} HCFU, respectively.

It is seen from Fig.8 for 37°C that the organelle structures are almost the same as those of the cells in the corresponding comparison group (Fig.4). But the spaces among the cells are present. Some cells contain more or deformed nuclei. The cell nuclei are big with a high nucleus/cytoplasm ratio.

As depicted in Fig.9 for 40°C, cells shrinkage, membrane blabbing, and circumferential condensation of the DNA at the nucleu membrane were seen. Spaces among the cells become larger with complete cell membranes. A lot of apoptosis bodies were observed. These morphological changes imply that the cells begin to present apoptosis.

From Fig.10 for 42.5°C, cell swelling, organelles disruption, and lysis were observed. These are typical features of cell necrosis.

It is clearly seen from Fig. 11 that COCL are completely dead at 43.5°C.

3.4. Quantitative Analysis through Image Processing

Maintenance of a stable cell volume under steady state condition is fundamental for normal cell function. On the other hand, change in cell volume plays an important physiological role as an integral part of the signal transduction events involved in such diverse processes as proliferation, apoptosis, migration, exocytosis, and neuronal excitability (Okada Y 2001; Pedersen SF 2002). Studies have indicated that cell volume decrease is one of significant characteristics of apoptosis. In addition, the measurement of the nucleus to cytoplasmic ratio (N/C) has become one of the important criteria in cancer clinical diagnose about the change level of tumour cells (Robinson LJ 1997; Lang F 2000).

Two issues need to be clarified here regarding the measurement of cell volumes. The first issue is how to define the cell volume. Another issue is how to calculate the volume from the obtained 2-dimensional TEM images. These two issues are discussed below.

Due to the difficulty in direct measurement of the absolute volume of a cell or a cell's structural component, the relative volume, i.e. volume density (usually in percentage), is usually measured instead. For the volumes of cell cytoplasm and nucleus, the whole cell can be considered to have the volume density of 100%, and both volumes of cytoplasm and nucleus are measured relative to the whole cell. For simplicity, the volume density is sometimes called volume in this work without confusion.

TEM images of cells display 2-dimensional cross-sections. According to Delesse principle, with ultrathin section, the ratio of the 2-dimensional areas of the sections of the structural components can be used to infer the ratio of their 3-dimensional volumes. This work employs this method to calculate the volume density. It is worth mentioning that the calculation of the cell volumes are carried out statistically through many TEM images (usually over 30), which are taken randomly.

In this experiment, cell volumes, nuclei volumes, and nucleus to cytoplasmic ratios were measured using the technique of image processing as discussed above. Average the calculation results for each of the comparison groups without addition of HCFU and the test groups with addition of HCFU. The quantitative results are tabulated in Table 1 and are graphically depicted in Figs. 12 and 13.

In Fig. 12, the results show that the cell volume in comparison group1 without HCFU changes significantly when temperature increases from 37°C to 40°C, and then remains steady when the temperature is beyond 40°C. In contrast, the cell volume in test group2 with HCFU changes slightly for the temperature between 37°C to 40°C, while sharply for the temperature beyond 40°C.

Fig.13 shows the changes in cell nuclei volumes. It is clearly seen that the cell nuclei volume in comparison group1 without HCFU decreases monotonously when the temperature increases from 37°C to 42.5°C. It is interesting to note that the cell nuclei volume of test group2 with HCFU remains almost constant for the temperature between 37°C and 40°C, while decreases sharply with the increase of temperature from 40°C to 42.5°C.

The calculated N/C results in table 1 show that the N/C ratios for both the comparison group1 without HCFU and test group2 with HCFU decrease as the temperature increases from 37°C and 40°C. Compared with the comparison group1 without HCFU, the test group2 with HCFU has a less significant change in N/C ratio for temperature below 40°C, and a more significant change in N/C ratio for temperature beyond 40°C.

The above results reveal that with the increase of temperature both the comparison and test groups display a decrease in cell volume, nuclei volume, and N/C ratio. Some changes are significant is these volumes and N/C ratio under certain conditions, especially for the temperature greater than 40°C. This implies that a higher temperature can induce the apoptosis of tumour cells, and the concurrent application of hyperthermia and chemotherapy can speed up the apoptosis of tumour cells. The quantitative results from image processing agree to those from microcalorimetry and TEM.

It is worth mentioning that previous sections have showed that the addition of HCFU can enhance the damage of tumour cells. This is evidenced by Figs. 12 and 13 for lower temperature (close to 37°C) and higher temperature (close to 42.4°C). However, no evidence has been found from these figures for a medium temperature at around 40°C. Further investigation is required to clarify this point.

4. Discussions

The induction of apoptosis of tumour cells has been investigated from both macroscopic and microscopic viewpoints. The macroscopic results obtained from microcalorimetry are verified by the microscopic results observed through TEM, and are partially evidenced by quantitative analysis of imaging processing.

The results of the TEM show that with the addition of drug and increase of temperature, human body carcinoma of ovary cell structures behave with more lethal changes. Typical changes are: cells shrinkage, bigger space among the cells, chromatin condensation, and membrane blabbing, nuclear fragments of varying size, loss of integrity of plasma membrane and organelles, and fragmentation into apoptosis bodies. When temperature is over 40°C, cell swelling, organellar disruption, and lysis are observed. These are typical characteristics of cell death. These changes lead to the obstruction of the material and energy metabolism of the tumour cells. This agrees to the microcalorimetric results, which show that the metabolism can become weaker, and eventually dead without metabolism.

The results from both TEM and microcalorimetry show that temperature, anti-tumour drug HCFU, and synergistic effect of both can all injure or even kill the human body carcinoma of ovary cells. This is accompanied with a series of morphological and energetic changes. The results of this work show that temperature and the anti-tumour drugs can cause both apoptosis and necrosis in the COCL.

It is also seen from this work that the combined employment of both the microcalorimetry and TEM techniques together with the quantitative analysis of imaging processing is a promising

methodology for investigation of the mechanism of tumour hyperthermia and tumour hyperthermia-chemotherapy.

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Captions of Illustrations

Table 1. Quantitative results of imaging processing.

Fig1. The power-time curves of COCL at 37°C, 40°C, 42.5°C, and 43.5°C, respectively.

Fig.2. COCL viable count versus time with and without 0.5μg.mL^{-1}HCFU and at 37°C, 40°C, 42.5°C, and 43.5°C, respectively.

Fig. 3 Power-time curves of COCL for comparison group without HCFU and test group with 0.5μg.ml^{-1} HCFU at different temperatures. (a) 37°C comparison and test groups. (b) 37°C and 40°C comparison groups, and 40°C test group. (c) 37°C and 42.5°C comparison groups, and 42.5°C test group. (d) 37°C and 43.5°C comparison groups, and 43.5^{0}C test group.

Fig. 4. TEM image of COCL cell of the comparison group (without HCFU) at 37°C (bar = 2μm).

Fig. 5. TEM image of COCL cell of the comparison group (without HCFU) at 40°C (bar = 2μm).

Fig. 6. TEM image of COCL cell of the comparison group (without HCFU) at 42.5°C (bar = 2μm).

Fig. 7. TEM image of COCL cell of the comparison group (without HCFU) at 43.5°C (bar = 2μm).

Fig.8. TEM image of COCL cell treated with HCFU at 37°C for 23 hours (bar = 2μm).

Fig. 9. TEM image of COCL cell treated with HCFU at 40°C for 23 hours (bar = 2μm).

Fig. 10. TEM image of COCL cell treated with HCFU at 42.5°C for 23 hours (bar = 2μm).

Fig.11. TEM image of COCL cell treated with HCFU at 43.5°C for 23 hours (bar = 2μm).

Fig.12. Changes of cell volume for comparison group1 without HCFU (solid line) and test group2 with HCFU (dotted line).

Fig.13. Changes of cell nuclei volume for comparison group1 without HCFU (solid line) and test group2 with HCFU (dotted line).

Table 1. Quantitative results of imaging processing.

	Comparison Groups (1)			Test Groups (2)		
	37°C	40°C	42.5°C	37°C	40°C	42.5°C
Cell volume (%)	12.9	9.5	9.6	11.6	10.8	8.1
Nuclei volume (%)	5.9	4.3	3.2	5.4	5.4	1.5
N/C ratio	1.102	0.939	0.643	0.972	0.944	0.228

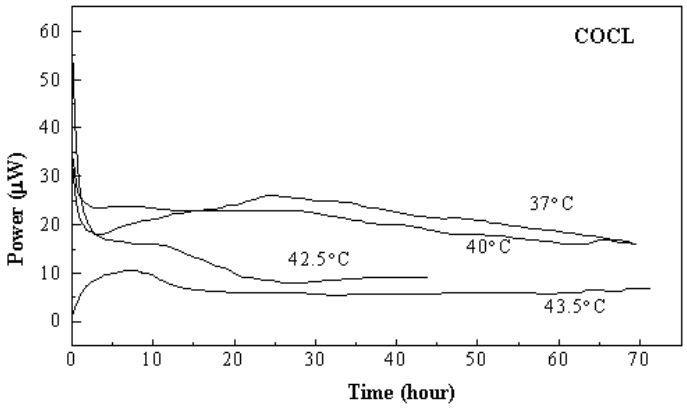

Fig1. The power-time curves of COCL at 37°C, 40°C, 42.5°C, and 43.5°C, respectively.

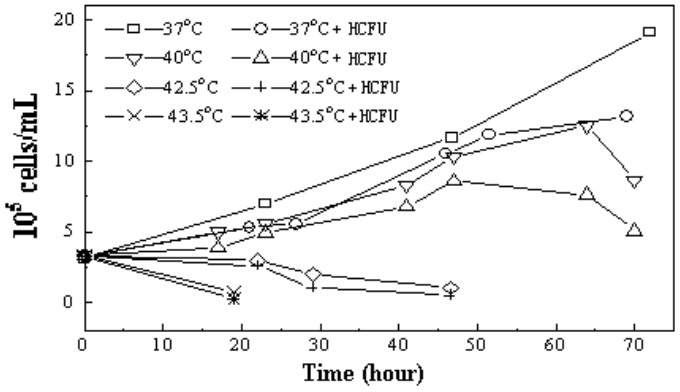

Fig.2. COCL viable count versus time with and without 0.5μg.mL^{-1}HCFU and at 37°C, 40°C, 42.5°C, and 43.5°C, respectively.

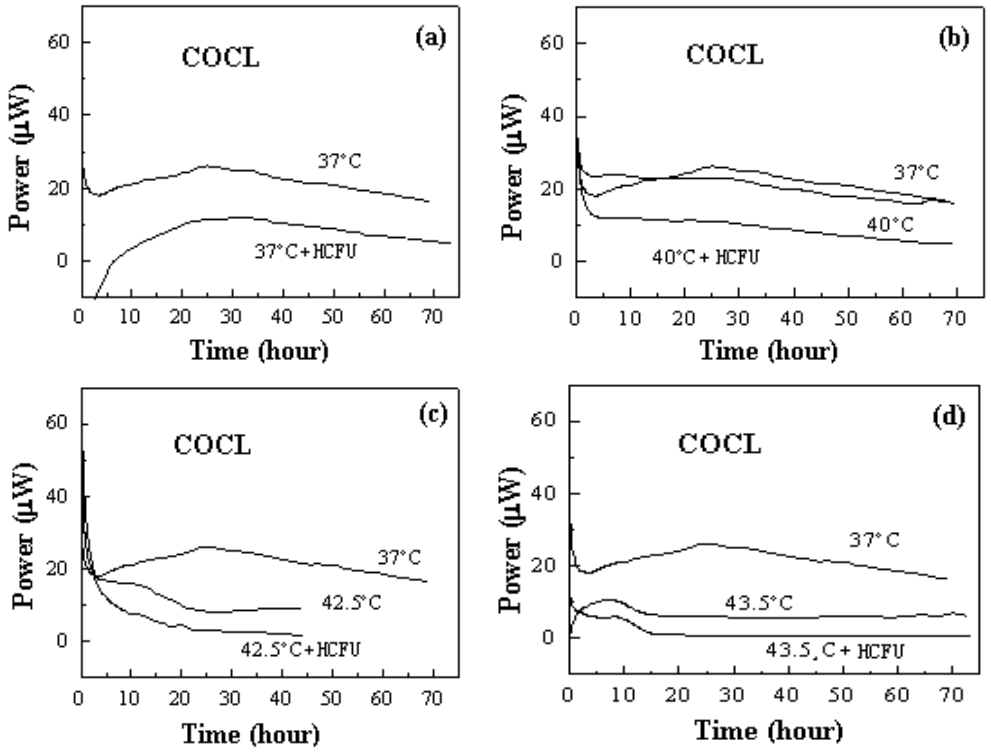

Fig. 3 Power-time curves of COCL for comparison group without HCFU and test group with 0.5μg.ml^{-1} HCFU at different temperatures. (a) 37°C comparison and test groups. (b) 37°C and 40°C comparison groups, and 40°C test group. (c) 37°C and 42.5°C comparison groups, and 42.5°C test group. (d) 37°C and 43.5°C comparison groups, and 43.5^{0}C test group.

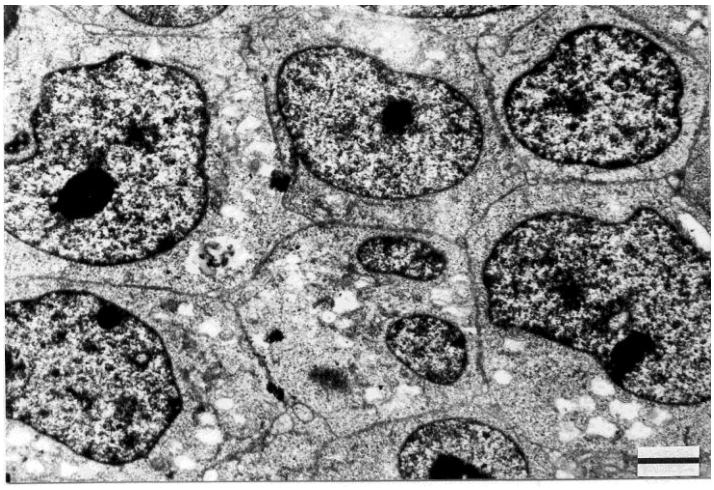

Fig. 4. TEM image of COCL cell of the comparison group (without HCFU) at 37°C (bar = 2μm).

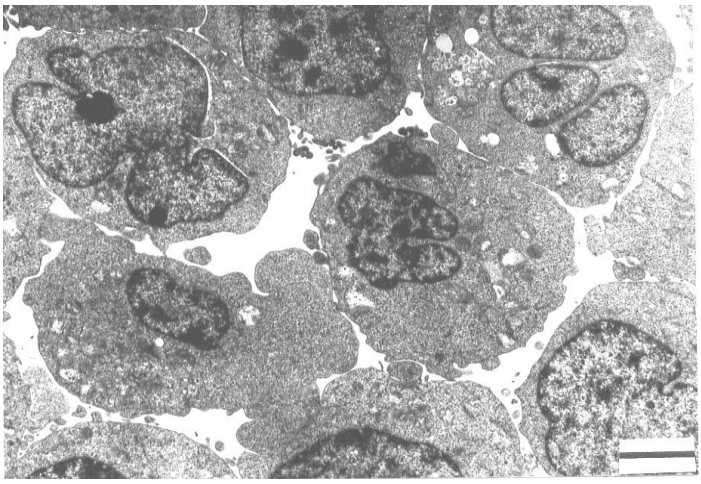

Fig. 5. TEM image of COCL cell of the comparison group (without HCFU) at 40°C (bar = 2μm).

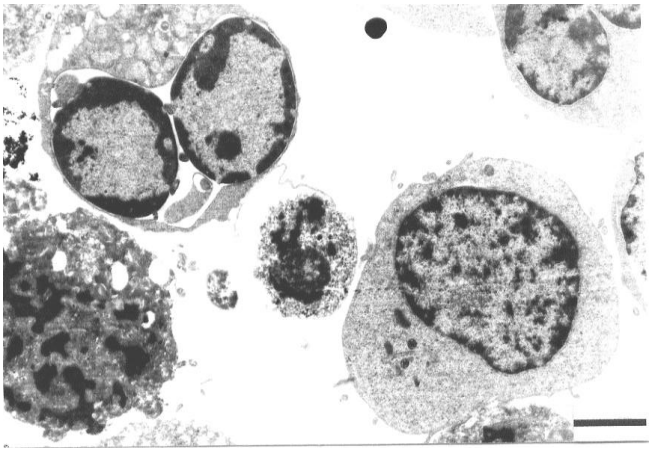

Fig. 6. TEM image of COCL cell of the comparison group (without HCFU) at 42.5°C (bar = 2μm)

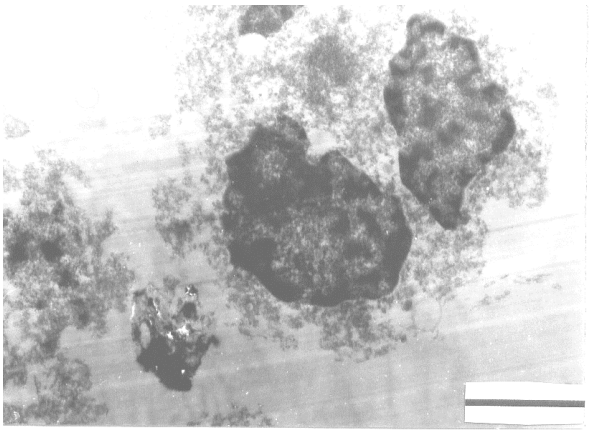

Fig. 7. TEM image of COCL cell of the comparison group (without HCFU) at 43.5°C (bar = 2μm)

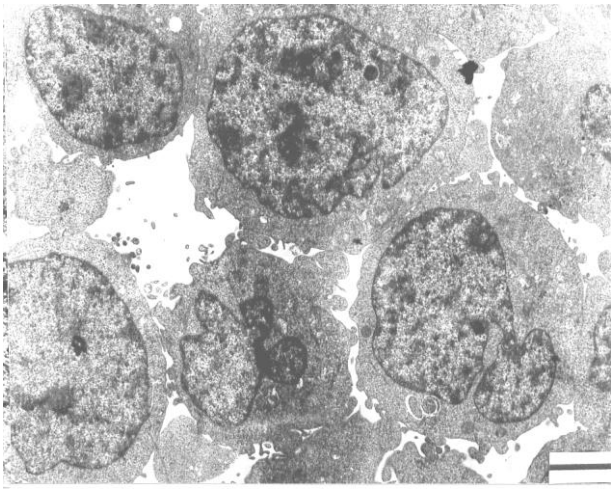

Fig.8. TEM image of COCL cell treated with HCFU at 37°C for 23 hours (bar = 2μm).

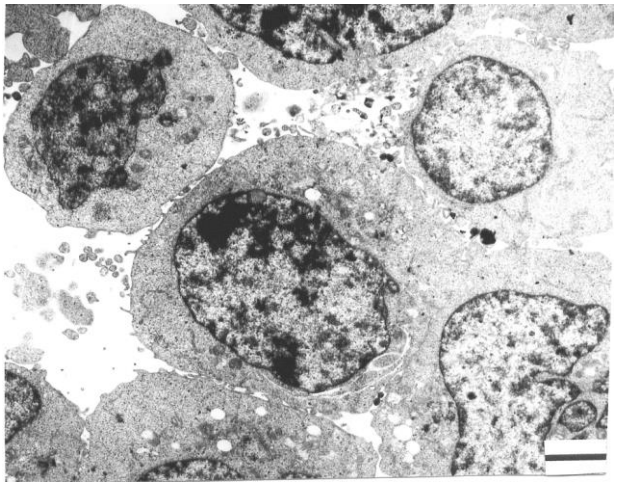

Fig. 9. TEM image of COCL cell treated with HCFU at 40°C for 23 hours (bar = 2μm).

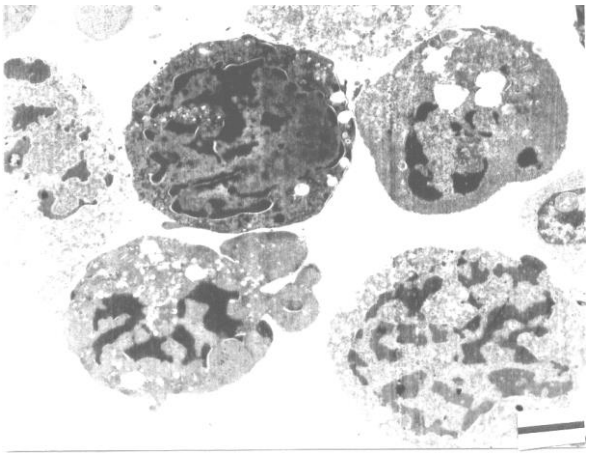

Fig. 10. TEM image of COCL cell treated with HCFU at 42.5°C for 23 hours (bar = 2μm).

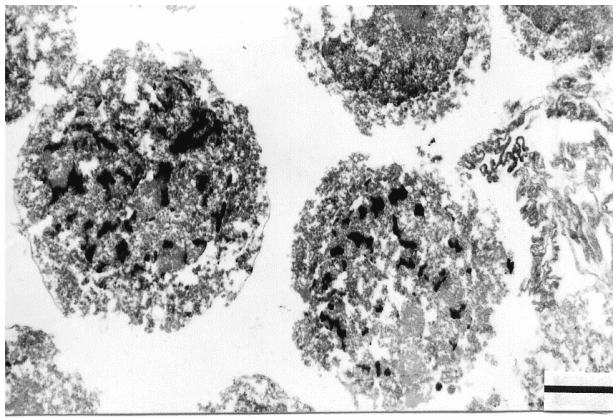

Fig.11. TEM image of COCL cell treated with HCFU at 43.5°C for 23 hours (bar = 2μm).

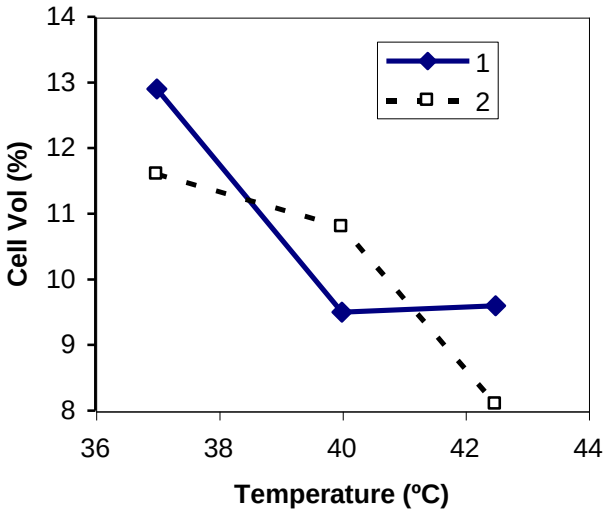

Fig.12. Changes of cell volume for ºcomparison group1 without HCFU (solid line) and test group2 with HCFU (dotted line).

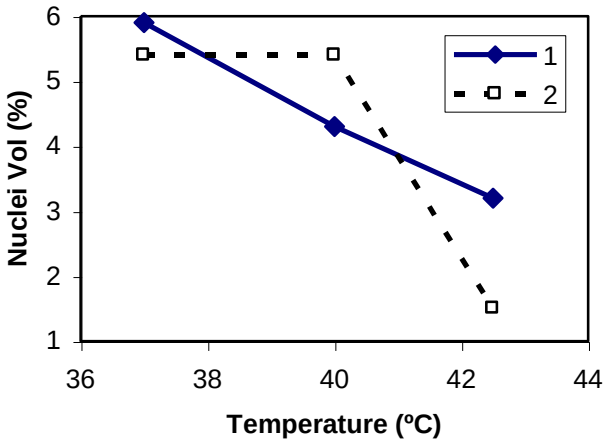

Fig.13. Changes of cell nuclei volume for comparison group1 without HCFU (solid line) and test group2 with HCFU (dotted line).